IMMOBILIZING AND PRECIPITATING ANTIGENS OF PARAMECIUM

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The pattern of inheritance of the immobilization antigens of *Paramecium aurelia* and the serological basis for the specific immobilization reactions have been studied by Sonneborn (1951), Beale (1952), van Wagtendonk and van Tijn (1953) and Finger (1955). The systems of transformation from one serotype to another have also been investigated (Sonneborn, 1950a; Beale, 1948) and possible mechanisms suggested (Kimball, 1947; Sonneborn, 1950b; Delbruck, 1949). Further work on the unique systems of inheritance and stability exhibited by the immobilization antigens has been hampered by the lack of a rapid, reproducible method for assaying them. We have now been able to detect these antigens through the development of a method which involves a modification of the techniques of Oudin (1952) and Oakley and Fulthorpe (1953). Specifically, a particular band formed by the diffusion of two reactants (antigen solution and antiserum) from opposite ends of an agar column has been identified as a complex, in part, of precipitated immobilization antigen and its homologous antibody. The present paper deals with the immobilization antigens and this *in vitro* method for detecting them.

Materials and Methods

Sera against whole animals prepared according to the methods described by Sonneborn (1950c) against several stocks of varieties 2, 4 and 8 were employed. These were tested with variety 2 antigen extracts of stock 3 and extracts of animals that were derived from the variety 2 stocks 7, 30 and 35. The survey of antigenic types found in variety 2, upon which much of the work reported here is based, has been submitted for publication. The antigen solutions were prepared in several ways: (1) Cilia were obtained from paramecia and extracted as follows (Preer and Finger, unpublished). Six million animals were concentrated by centrifugation and one volume placed in twenty volumes of 0.22% sodium chloride buffered at pH 7.8 with 0.01 *M* sodium phosphate. At the end of ten minutes the animals were centrifuged lightly at 890 *g* for two minutes; the supernatant (containing mainly cilia) was retained and again centrifuged lightly. Any sediment of animals and trichocysts formed was then removed and the process repeated until the supernatant was free of animals. The supernatant was then centrifuged for two minutes at 24,000 *g* in order to sediment the cilia. Then one ml. of 0.9% sodium chloride, buffered to pH 7.0 with 0.01 *M* sodium phosphate, was added to the centrifugate. After 24

[1] Philadelphia Brewers Association Fellow, 1954–55. This work was also supported by a grant from the Phi Beta Psi Sorority administered by Dr. John R. Preer.

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hours at room temperature this suspension was centrifuged at 24,000 *g* for two minutes and the clear supernatant used as antigen. (2) Lyophilized animals were placed in a 0.9% sodium chloride solution overnight in a refrigerator, centrifuged at 24,000 *g* for two minutes and the supernatant used as antigen. (3) Animals were concentrated by centrifugation, placed in a deep freeze, and, after thawing at room temperature, the entire brei was used as antigen. (4) A brei was made by repeatedly forcing living animals from a syringe against the walls of a glass cylinder at room temperature. The resulting homogenate was then centrifuged for ten minutes at 24,000 *g* and the supernatant used as antigen. These four preparations were not equally effective in yielding the immobilization antigen and are listed in increasing order of efficiency. When quantitatively comparable data were desired the antigen solutions used were, of course, obtained by the same method.

The method of gel diffusion has been given in detail by Preer (1956). It is as follows: Pyrex tubes, approximately 2-mm. inside diameter, were coated with a 0.1% agar solution, evacuated, cut into 4-cm. lengths and flame-sealed at one end. To set up a double-diffusion test 0.01 ml. of antiserum was added with a syringe to a tube held upright in a Cartesian diver loader. Then 0.6% washed, merthiolated, and buffered (pH 7.0) agar maintained at 60° C. was carefully layered with a warm syringe until an agar column of 6–8 mm. height was reached. A third layer of 0.01 ml. antigen solution was placed on the agar after it had solidified. The tube was then sealed with Picene cement and placed in a horizontal position at 24° C. When serum dilutions were used, normal serum was used as the diluent. This was to prevent mixing of antiserum with the warm agar at the time of layering. The diluent for the antigen was usually 0.9% sodium chloride or, less frequently, 10% Ringer's solution.

The location of a zone of precipitation within the agar was used as a measure of the concentration of one of the reactants (antigen or antibody) contributing to the band when the concentration of the other reactant was kept constant (Preer, 1956). The position of a band was measured after 24–72 hours with a binocular dissecting microscope provided with an eyepiece micrometer.

Variations on these general techniques, *e.g.*, mutual dilution, absorption experiments, etc. are described below.

Results

When a solution of several antigens is diffused against homologous antiserum, bands of precipitate will be formed as a result of the specific complexing of antigens with antibody. Because the position of a band is dependent on several factors (concentration of reactants, diffusion coefficients, etc.), each serologically distinct precipitating system usually appears as a separate zone of precipitation. Occasionally, however, two or more precipitating systems may, through a fortuitous combination of these factors, appear as a single band. Therefore the number of bands will represent the minimum number of antigen-antibody systems present.

Many workers have demonstrated that *P. aurelia* may manifest a number of different antigenic types, called "serotypes," and designated by the letters A, B, C, etc. When animals of a given serotype are placed into a suitable dilution of antiserum prepared against that type, their locomotion slows, and they become immobilized. Each serotype is serologically distinct from any of the others, an animal

of a particular serotype generally becoming immobilized only when placed in homologous antiserum. If a number of antisera are allowed to diffuse against extracts of homologous serotypes and against extracts of heterologous serotypes, then it should be possible to correlate the presence of certain bands with these specific immobilization antigen-antibody systems.

When animals of the G serotype were used as the source of antigens and G antiserum diffused against the antigen solution, a band formed that was not present when an antigen solution prepared from non-G animals reacted with a G antiserum. Six different stocks of *P. aurelia*, all of serotype G, possessed a similar antigen, an antigen absent in extracts of two stocks of a serotype other than G and in non-G animals of two other species, *P. caudatum* and *P. polycaryum*.

Analogous results were obtained with C antigen-antiserum systems. A band appeared in these homologous systems that was missing when the antigen solution was obtained from non-C animals.

To provide further evidence that this antigen was found only in homologous serotypes, absorption experiments were performed. It was found that extracts of several stocks manifesting the G serotype were able to remove the specific precipitin from G antisera while several stocks of a different serotype and non-G *P. caudatum* and *P. polycaryum* preparations were ineffective.

The precipitating antigen associated with a particular serotype was further homologized between stocks through the use of a mutual dilution technique (Oudin, 1952; Telfer and Williams, 1953). Although all antigen preparations were able to form a distinctive band when diffused against homologous antisera, it was possible that the bands formed with different antigen preparations did not represent the same antigen-antibody systems, the correspondence being coincidental. An antigen forming a band against a particular serum may be identified with an antigen from a second extract by mutually diluting the two extracts, one with the other, and noting whether the bands formed behave independently, as though diluted with a neutral reagent, or act to reinforce each other. In order to employ the mutual dilution technique with maximum effectiveness, the concentrations of serum and antigen solution were chosen so as to eliminate most or all of the bands in the system, aside from the ones being compared, and still leave a band intense enough to withstand a two-fold dilution of antigen. For optimum resolution in a mutual dilution series the concentrations were adjusted so that the bands in the two preparations being compared formed at widely separated positions in the original undiluted systems. As a result of the mutual dilution studies, the bands formed with different preparations of the same serotype were shown to represent the same antigen-antibody system.

In summary, then, a study of serotypes G and C has shown that animals of each serotype have a specific precipitating antigen which is lacking in animals of other serotypes. It seems likely, then, that these antigens are the immobilizing antigens. Such an immobilization antigen can be identified in gel-diffusion systems in several ways: (1) a comparison of bands formed with homologous and heterologous antigens; (2) specific absorption by heterologous extracts of all antibody from a homologous antiserum, but the one that forms a zone of precipitation with homologous extracts; and (3) the mutual dilution of an extract with an antigen solution which forms several bands, one of which is known to be formed by the specific precipitinogen.

Although the immobilization antigen and the specific precipitating antigen are probably the same, there is evidence that the precipitated band may not be composed solely of immobilizing antigen and immobilizing antibody. The evidence has been obtained from a comparison of immobilization titers of antisera and precipitin titers as measured by the specific band position.

This specific band was identified as above by diffusing antisera against antigens extracted from two clones with the same genotype but of contrasting serotypes. The eleven sera titered for antibody forming this band were prepared against several different stocks and were known to immobilize animals of the G serotype in concentrations of 1 : 12.5 or less after two hours at room temperature. Eight of these anti-G sera, all having an immobilization titer of 1 : 50 or greater, precipitated

TABLE I

Comparison of immobilization titers and gel diffusion band titers of antisera against P. aurelia

Serum	Anti-G titer	G-precipitin titer	Anti-C titer	C-precipitin titer	Serum	Anti-G titer	G-precipitin titer	Anti-C titer	C-precipitin titer
P#23	100	40	0	0	F#10	0	0	100	100
P#16	100	9	0	0	P#8	0	0	100	65
P#2	75	100	0	0	F#17	0	0	50	30
P#12	75	100	0	0	F#11	0	0	40	90
P#7	50	15	0	0	F#4	0	0	25	25
P#14	50	30	0	0	F#5	0	0	25	45
P#17	50	15	0	0	F#12	0	0	20	9
F#15	20	45	15	4	P#18	0	0	15	4
F#18	15	3	0	0	F#3	0	0	15	7
F#14B	5	5	20	11	P#19	0	0	5	7
F#13B	3	1	0	1	F#1	0	0	3	0
					P#4	0	2	0	0
					P#21	0	3	0	0

The immobilization antibody titers (anti-G and anti-C titers) and the gel diffusion precipitin titers are presented on a scale with sera having the greatest concentration of antibody listed as 100, and the antibody concentration of all other sera, as compared with these sera, being denoted by numbers from 0–100. In this way, a serum with half the antibody content of the strongest sera would be given the number 50, etc. The titers presented above represent the means of several series of titrations.

a single band against the G antigen solution that was absent when preparations of the C serotype were used as reactants. Of the three anti-G sera that showed the same number and type of bands with both G and C antigen solutions, two had immobilization titers against both serotypes (F#15 and F#14B) and one (F#13B) had negligible anti-C precipitin titer. These data are presented in Table I where the immobilization titers of 24 antisera against the G and C serotypes are compared with the precipitating antibody concentration as determined by band position.

Similar results were obtained with anti-C sera when the sera were titered against homologous and heterologous solutions. Ten of thirteen antisera gave a band with C antigen solutions not present when G antigen solutions were used. Of the three homologous antisera that did not precipitate this band, one had a very low immobilization titer (F#1), and two also had anti-G immobilization titer. Thus, any differences that may have existed due to the C immobilizing antibodies

would be obscured (F#15 and F#14B). Seventeen sera prepared against several serotypes (omitted from Table I) and with neither G nor C immobilization antibody presented essentially identical band series with G and C antigen solutions.

Following the procedure used in the studies on antigens, absorption experiments were employed to confirm the identification of the immobilization antibody. The standard G antiserum was diffused against a G antigen extract that had previously been incubated with antiserum at 12° C. for 24 hours. Nine sera which immobilized G animals in two hours when used in dilutions of 1 : 200 or greater successfully absorbed the antigen responsible for the band found in G antigen-antibody systems. Three with poor G titers and 26 without any G titer when used as absorbents had no effect on the appearance or position of the band.

A final corroborative group of experiments was carried out using the mutual dilution method. A single serum having a high immobilization titer against G animals and precipitating antibody capable of withstanding several-fold dilution was chosen as a standard serum. When eight anti-G sera were mutually diluted with the standard serum, it was found that the antibody restricted to the G antigen-antibody reaction and present in all systems involving these sera was either related to or identical with the antibody found in the standard G antiserum, the band formed being reinforced upon the addition of the sera being tested. Additional mutual dilutions among the eight sera confirmed this finding.

Although the evidence for the identity of the band characteristic of homologous systems with the immobilization antigen and antibody is convincing, there appears to be a rather poor correlation between the titer of a serum as determined by immobilization tests and the concentration of precipitating antibody. Thus, serum P#16 with about the same G immobilization titer as sera P#23, P#2, and P#12 has less than 25% of the precipitating antibody of these antisera (Table I). Other "exceptional" sera are P#4 and P#21 which possess precipitating antibody and yet do not immobilize. It is apparent, then, that immobilization antibody and precipitating antibody, although closely correlated, are not identical. The serological nature of the relationship of the two kinds of antibodies is being studied. As would be expected, preliminary absorption experiments have demonstrated that it is possible to remove all precipitating antibody from certain antisera without abolishing all immobilizing activity.

As for the immobilization antigens, although these studies have demonstrated that in animals of serotype G one particular precipitating antigen is found and in animals of serotype C it is not found, but a second precipitating antigen is, it is not known whether immobilization antigens and precipitating antigens are identical. It is possible that only a portion of the immobilization antigen is capable of precipitation or that there may be precipitating antigen that does not take part in immobilization.

Summary

1. A study of serotypes G and C, of variety 2, *Paramecium aurelia,* has been made, using diffusion in agar. It has been shown that animals of each serotype have a specific precipitating antigen which is lacking in animals of other serotypes. Consequently, it seems probable that these antigens are the immobilizing antigens.

2. Comparisons of antibody concentration, as determined by immobilization titers and by band position, show that the precipitated band may not be composed

solely of immobilizing antigen and antibody, and that there is precipitating antibody that is not capable of immobilizing.

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